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Evaluation of Gentamicin for Use in Virology and Tissue Culture

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Data are presented comparing gentamicin to penicillin and streptomycin (Pen-Strep) in tissue culture medium with respect to a number of parameters associated with virology and tissue culture. Unlike Pen-Strep, gentamicin was stable at *p*H 2 to 10 for 15 days at 37 C in tissue culture medium, and its activity was unaffected by the presence of serum. Moreover, it was stable to autoclaving. Twenty cell types replicated normally at the suggested concentration of 50 μg/ml, and all cells were unaffected by 20 times this concentration. Evidence for its practical use in virus studies was demonstrated in that (i) it was not viricidal to ribonucleic acid or deoxyribonucleic acid viruses at 40 times the suggested concentration at 37 C, (ii) the size and number of plaques were not affected by 20 times the suggested concentration, (iii) interferon assays and production were unaffected by 20 times the suggested concentrations. Gentamicin may be uniquely useful for shipment of clinical specimens and long-term tissue culture and virus studies.

Penicillin and streptomycin (Pen-Strep) have been used in cell culture for years in spite of several undesirable characteristics of the mixture. Streptomycin is rapidly destroyed at alkaline *p*H, and penicillin is extremely unstable to both acid and alkaline *p*H. Neither antibiotic can withstand autoclaving, and the activity of penicillin is reduced in the presence of serum. Neither has as wide a spectrum of biological activity as gentamicin.

Occasionally antibiotics which exhibit low cytotoxicity are introduced for use in cell culture studies (e.g., tylosin, bacitracin, kanamycin). However, little effort has been directed towards determining the effects of these antibiotics on several important parameters often measured by the virologist. These parameters include the influence of the antibiotic on cell multiplication, virus replication, virus yield, virus infectivity, interferon production, interferon assays, and numerous other indexes of biological activity.

Gentamicin has biological and biochemical properties which may render it superior to penicillin alone, streptomycin alone, or the two in combination for use in tissue culture. It is active against strains of *Proteus* and *Staphylococcus* as well as numerous other gram-positive and gram-negative organisms (1). Moreover, unlike Pen-Strep, gentamicin is active against the ubiquitous *Pseudomonas* strains (1, 13). When used in tissue culture at the suggested concentration of 50 μg/ml (base equivalent), it is bactericidal for a wide variety of bacteria. Although it is not active against yeasts, fungi, or protozoa, it has been reported to be active against several strains of *Mycoplasma* (2, 9, 10). These characteristics make gentamicin a candidate for use in cell and tissue culture and also for transport of cell cultures, certain clinical specimens containing viruses, or specimens in which overgrowth by bacteria should be avoided.

The purpose of this study was to evaluate and compare gentamicin in cell cultures with Pen-Strep by studying the influence of these antibiotics on several parameters of common interest to those who employ tissue culture techniques. Data are also presented on the effects of gentamicin on cell morphology and multiplication, virus stability and replication, and the synthesis and assay of interferon. An investigation of the antibacterial activity of gentamicin was not conducted as such studies have been already reported (4, 6, 11).

MATERIALS AND METHODS

Gentamicin solution. Gentamicin solution was reconstituted in distilled water equivalent to 10,000 μg of gentamicin base per ml, passed through a 0.22-

μm membrane filter (Millipore Corp.), and stored at room temperature. The stock solution was diluted to 50 μg/ml in tissue culture media, unless specified otherwise. A concentration of 50 μg/ml was chosen on the basis of studies in which the antibiotic potency (minimal inhibitory concentration) of gentamicin was determined against 14 different species of bacteria (14). Gentamicin consists of several closely related isomeric pseudotrisaccharides and is also referred to as gentamicin complex. It has an average molecular weight of 463. It is available as Gentamicin Reagent Solution (Schering Corp.) and should be distinguished from Garamycin (Schering Corp.). The latter contains, in addition to gentamicin sulfate, methylparaben and propylparaben (preservatives), sodium bisulfite (reducing agent to prevent discoloration), and disodium ethylenediamine-tetraacetate (chelating agent). Garamycin is indicated for clinical use, whereas Gentamicin Reagent Solution is intended for use in tissue culture studies.

Other antibiotics. Penicillin G-streptomycin sulfate solution (10,000 units of penicillin and 10,000 μg of streptomycin/ml) was obtained from Grand Island Biological Co., Grand Island, N.Y.

Tissue culture medium. In nearly all experiments reported here, McCoy's 5A medium and Medium 199 were employed as representative media commonly used by many laboratories.

Viruses. Ribonucleic acid (RNA)- and deoxyribonucleic acid (DNA)-containing viruses used in this study included Sindbis virus (strain HR), the Indiana strain of vesicular stomatitis virus (VSV), type 2 (HGP) rhinovirus, Newcastle disease virus (NDV), herpes simplex virus, and vaccinia virus.

Cells. Several cell types were used for virus assay and propagation. These included chick embryo fibroblasts (CEF) and HeLa (Rhino) cells obtained from Grand Island Biological Co., Grand Island, N.Y., and mouse fibroblasts (L-929).

Virus assays. All viruses were quantified by plaque assay. Overlay medium consisting of Eagle's minimal essential medium (MEM) containing 3% fetal calf serum, 0.07% $NaHCO_3$, 0.8% agarose, and 50 μg gentamicin per ml was applied to infected monolayers in petri plates (Falcon). Plates were incubated at 37 C for 2 to 3 days for plaque development and were stained and counted on day 3 or 4. Rhinovirus was assayed on HeLa (rhino) monolayers. The overlay consisted of McCoy's 5A modified with 3% fetal calf serum, 30 mM $MgCl_2$, 50 μg of gentamicin per ml, and 0.6% agarose. Plates were incubated at 34.5 C for 2 days, and plaques were stained and counted on day 3.

Effect of gentamicin on virus replication. Monolayers on 60-mm petri plates (Falcon) were washed once with Dulbecco's phosphate-buffered saline (PBS), infected with 0.5 ml of virus in PBS, and placed at 37 C. One hour later the monolayers were washed three times with PBS followed by the addition of 3 ml of MEM containing various amounts of gentamicin or Pen-Strep and 3% fetal calf serum and incubated at 37 C (rhinovirus-infected cells were incubated at 34.5 C). After 24 to 48 hr, cells and fluids were harvested and stored at −60 C for assay.

Effect of gentamicin on interferon synthesis. Monolayers of L-929 cells were inoculated with egg-propagated NDV at a multiplicity of 10 plaque-forming units (PFU)/cell and adsorbed for 1 hr, and the residual inoculum was aspirated. Petri plates were incubated at 37 C with 3.5 ml of MEM containing 0.5% bovine serum albumin and various concentrations of gentamicin or Pen-Strep. After 24 hr, the fluid was harvested, dialyzed against 20 volumes of HCl-KCl buffer at *p*H 2 for 5 days at 4 C followed by dialysis against PBS at *p*H 7 for 3 days at 4 C, passed through a 0.45-μm filter, and stored at −60 C.

Effect of gentamicin on interferon assays. Interferon was assayed by the plaque inhibition test described by Wagner (12) employing L-929 cells and VSV. Interferon titers were expressed as the reciprocals of the dilution of 1.0 ml of fluid which inhibited 50% of VSV plaques. Assays were conducted in the presence and absence of gentamicin or Pen-Strep.

Stability of antibiotics at various pH levels. Either gentamicin (50 μg/ml final concentration) or Pen-Strep (100 units and 100 μg/ml final concentration, respectively) was added to a commonly used tissue culture fluid, Medium 199 containing 3% fetal calf serum. The *p*H was adjusted with 5 N NaOH or 6 N HCl and thoroughly mixed, and 1.5 ml of the complete medium was dispensed into glass ampoules, immediately flame-sealed, and placed at 37 C. At various time intervals, ampoules were frozen at −60 C pending assay for antibiotic activity.

Antibiotic assays. Gentamicin was quantitated by the method of Oden et al. (8). Penicillin and streptomycin were assayed by the methods described by Grove and Randall (5).

RESULTS

pH stability of several antibiotics in cell culture medium. The stability of gentamicin at various *p*H levels was determined and compared with *p*H stability of Pen-Strep under identical conditions in Medium 199 containing 3% fetal calf serum. Medium was adjusted to the indicated *p*H (Fig. 1), and antibiotic was added and dispensed as described (see above). Samples were analyzed for biological activity as described above.

Gentamicin was found to be completely stable at all *p*H levels. Streptomycin showed progressive loss of activity at *p*H 6.5, 7.0, 7.5, 8.0, and 10.0, and penicillin lost activity more rapidly than did streptomycin at all *p*H levels. Furthermore, it was observed that penicillin immediately lost at least 30% of its activity upon addition to the medium containing serum. The decrease of penicillin activity may be attributed to the complexing of the antibiotic with serum proteins.

Antibiotic stabilities to autoclaving. Since tissue culture medium which can be autoclaved is now available, it would be advanta-

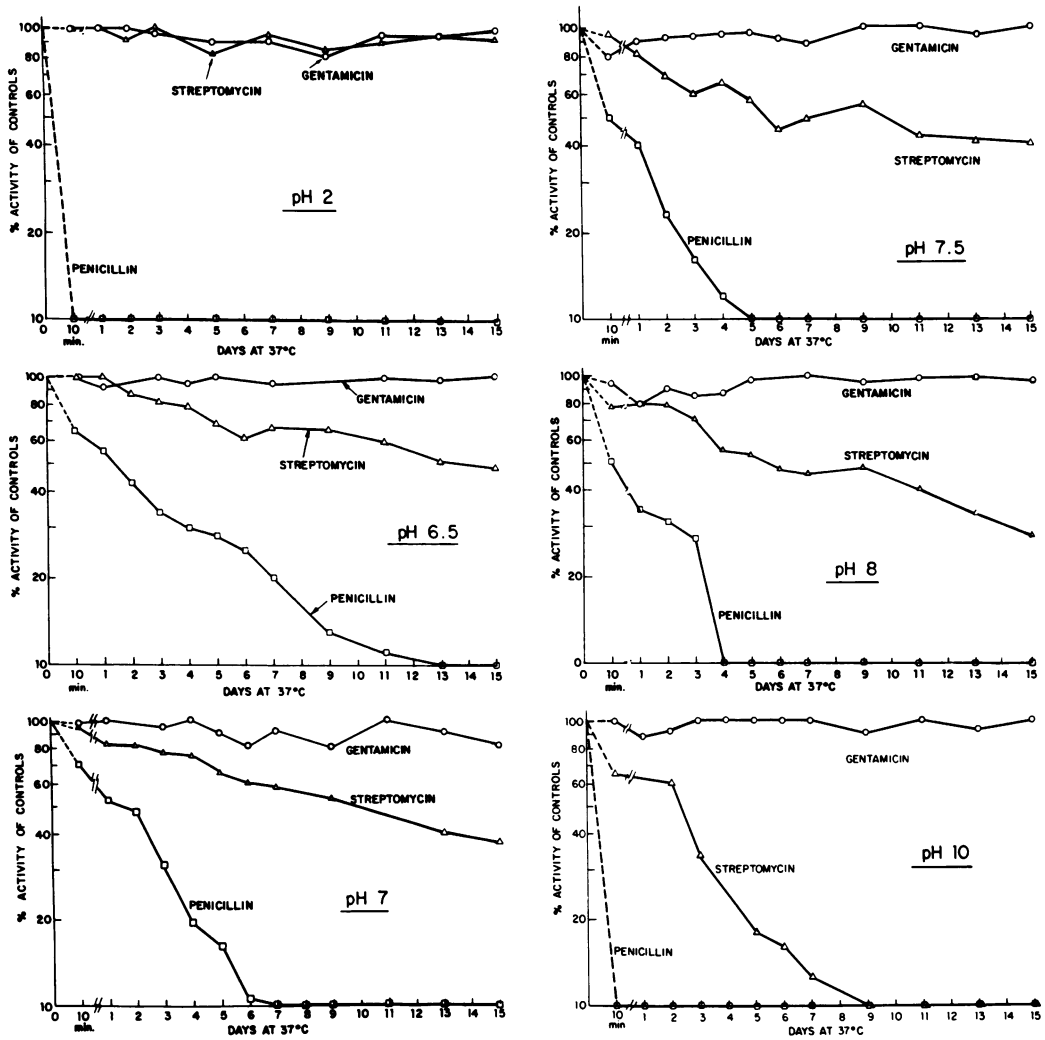

FIG. 1. *Stability of antibiotics in Medium 199 at various pH levels. Initial concentration were: 50 μg of gentamicin per ml, 100 units of penicillin per ml, and 100 μg of streptomycin per ml. For control purposes, samples of the antibiotics were taken immediately after addition to medium and stored at −70 C pending assay.*

geous to add antibiotic to the medium before autoclaving. To determine the influence of autoclaving on antibiotics, the following experiment was performed. Aqueous solutions of penicillin (100 units/ml), streptomycin (100 μg/ml), or gentamicin (50 μg/ml) were prepared. Half of each sample was stored at 4 C for control purposes, whereas the other half of each sample was autoclaved for 15 min at 121 C and 15 lb of pressure. Under these conditions, penicillin was almost completely inactivated (99%) and streptomycin activity was reduced by 50%. In contrast, gentamicin was completely stable.

Cell tolerance to gentamicin. Twenty cell types of various origins were passaged continuously at least 20 times in the presence of 50 μg of gentamicin per ml (Table 1). Usually, at each passage level, cells were fed with fresh medium two or three times at intervals of 1 to 4 days. No microscopically detectable changes in cell morphology nor changes in growth characteristics were observed.

In another series of studies, seven commonly used cells were selected to determine the maximum tolerated dose of gentamicin. Monolayers on 60-mm petri dishes were incubated with various concentrations of gentamicin or

with antibiotic-free medium and microscopically examined daily for morphological changes (Table 2). AC amnion cells incubated for 4 days with medium containing antibiotic showed no gross morphological changes. This concentration is 20 times the suggested dose of gentamicin for use in cell culture. As indicated in the last column, 6 mg of gentamicin per ml was required to produce gross morphological alterations of AC amnion cells by day 2.

TABLE 1. *Cell types continuously[a] passaged in gentamicin*

Origin	Cell line
Human	AC amnion
	Human fetal trachea
	M-HeLa
	R-HeLa
	HEp-2
	U-amnion
	FS-1 foreskin
Hamster	BHK-9
	BHK-21/C13
	HKCC
	DON
	Ha K
Mouse	L-929
Feline	Feline lung
	CRFK Crandell feline kidney
Monkey	MK 2
	Vero
Fish	Fat head minnow
Bovine	MDBK
	EBTr

[a] Cells were passaged at least 20 times in gentamicin, 50 μg/ml.

Hence, the suggested concentration for tissue culture use was found to be well below the maximum tolerated dose for all seven cell types as evidenced by the lack or morphological alterations.

Stability and replication of viruses in the presence of gentamicin. Although gentamicin was well tolerated by cells, it was of interest to determine its affects on virus stability and replication. To this end, selected representatives of some of the major classes of RNA- and DNA-containing viruses were incubated with 2 mg of gentamicin per ml in PBS, far in excess of the suggested concentration, or with PBS free from antibiotic at 37 and 4 C. Samples were removed at 1 and 4 hr after the addition of gentamicin, and virus infectivity was determined by plaque assay. The results shown in Table 3 indicate that the infectivity of all viruses tested was unaffected by 40 times the suggested concentration of gentamicin.

To determine the effect of the suggested concentration of gentamicin (50 μg/ml) upon virus replication, duplicate monolayers were infected with the viruses listed in Table 4 and incubated with medium containing Pen-Strep, gentamicin, or antibiotic-free medium. Cytopathology developed at the same rate in monolayers with antibiotic-free or antibiotic-containing medium. Supernatant fluids were harvested after most of the cells had been destroyed and virus yields were determined. It can be seen that gentamicin did not affect virus yields.

Other experiments demonstrated that 1 mg of gentamicin per ml incorporated into the agar overlay had no effect on the number or the size of VSV plaques in chick embryo fibroblasts. Size and number of plaques were markedly reduced only at extremely high concentrations (5 mg/ml).

TABLE 2. *Effect of gentamicin on cell cultures*

Cell culture	Nontoxic concn[a] (μg/ml)	Tolerated:suggested[b] dose	Toxic concn[c] (μg/ml)
Human amnion (AC amnion)	1,000 (4)	20×	6,000 (2)
Chick fibroblast	1,000 (5)	20×	3,000 (3)
Human foreskin (FS-1)	1,000 (6)	20×	3,000 (2)
Human carcinoma of cervix (HeLa)	6,000 (3)	120×	
Mouse fibroblast (L-929)	1,000 (3)	20×	6,000 (2)
Human amnion (U)	6,000 (3)	120×	
Monkey kidney (Vero)	4,000 (4)	80×	5,000 (2)

[a] Number of days observed noted in parentheses.

[b] Tolerated dose was determined experimentally. The suggested dose was arbitrarily selected on the basis of in vitro antibacterial activity.

[c] Numbers in parentheses indicate day at which gross morphological changes were observed.

TABLE 3. *Stability of RNA and DNA viruses to 2,000 μg of gentamicin per ml in phosphate-buffered saline (PBS)*

Virus	Plaque-forming units/ml			
	37 C/1 hr	4 C/1 hr	37 C/4 hr	4 C/4 hr
RNA viruses				
Sindbis (HR)				
Gentamicin	1×10^8		1.5×10^8	
PBS	1.2×10^8	1.4×10^8	1.4×10^8	2.3×10^8
Vesicular stomatitis virus				
Gentamicin	2.3×10^8		2.8×10^8	
PBS	2.8×10^8	2.5×10^8	4.2×10^8	5.9×10^8
Rhinovirus (HGP)				
Gentamicin	6.2×10^7		6.4×10^7	
PBS	9.5×10^7	9.6×10^7	6.7×10^7	1.1×10^8
DNA viruses				
Herpes simplex				
Gentamicin	1.8×10^6		1.3×10^6	
PBS	1.1×10^6	1.7×10^6	1.1×10^6	1.8×10^6
Vaccinia				
Gentamicin	2.8×10^6		2.4×10^6	
PBS	2.7×10^6	2.8×10^6	2.3×10^6	2.6×10^6

TABLE 4. *Effect of gentamicin on virus replication*

Virus	Virus yields (PFU/ml)[a]		
	No antibiotic	Pen (100 units/ml)-Strep (100 μg/ml)	Gentamicin (50 μg/ml)
RNA viruses			
Rhinovirus (HGP)	1.6×10^7	1.7×10^7	1.7×10^7
Newcastle disease virus	2.0×10^7	2.1×10^7	3.4×10^7
Sindbis (HR)	2.9×10^8	4.4×10^8	3.4×10^8
Vesicular stomatitis virus	7.3×10^8	4.9×10^8	7.9×10^8
DNA viruses			
Herpes simplex	1.9×10^7	3.2×10^7	1.4×10^7
Vaccinia	8.0×10^5	6.1×10^5	7.6×10^5

[a] PFU, plaque-forming units.

Effect of gentamicin on interferon synthesis. L-929 cells were stimulated to synthesize interferon with live NDV as described (see above) in the presence and absence of gentamicin (1 mg/ml). Under these conditions, interferon yields were not affected.

Effect of gentamicin on interferon assays. The influence of gentamicin on the sensitivity of the interferon assay was studied by assaying mouse serum interferon in the presence of several concentrations of gentamicin. Serum containing interferon was diluted in medium with either 50, 100, 500, or 1,000 μg of gentamicin per ml and incubated with monolayers at 37 C. After 24 hr, the monolayers were washed three times and challenged with 50 to 100 PFU of VSV and overlaid with agarose. Concentrations as great as 1,000 μg/ml had no affect on the assay results. Higher concentrations were not tested. Thus, the sensitivity of interferon assays was unaffected by 20 times the suggested concentration of gentamicin.

DISCUSSION

These and other studies indicate that gentamicin offers several advantages over Pen-Strep for use in cell culture. The data confirmed Casemore's observation (4) which showed that gentamicin was completely stable to autoclaving and thus could be added to media before sterilization. The microbiological activity of gentamicin was completely stable in cell culture medium over a wide range of *p*H and temperature. These characteristics are especially desirable since cells, viruses, and clinical

specimens may undergo wide fluctuations in *p*H and temperature while in transit or during long-term experiments. The stability of gentamicin at *p*H 2 is of added value, since interferon preparations are frequently subjected to *p*H 2 for 1 to 5 days to preferentially inactivate virus.

As much as 85% of some types of penicillin are bound by human serum (3). Similarly, 30% of the biological activity of penicillin G was lost immediately when it was added to tissue culture medium containing 3% fetal calf serum (Fig. 1). The biological activity of gentamicin was not reduced in the presence of serum.

An ideal antibiotic for cell culture should have broad-spectrum antibacterial and antimycoplasma activity as well as freedom from cell toxicity. Gentamicin approaches these requirements. Casemore (4) reported that it was not toxic to human amnion cells, rhesus monkey cells, or HeLa cells at a concentration of 50 μg/ml, and Rudin et al. (11) indicated that toxicity was not observed with African green monkey kidney cells or WI-38 cells at a concentration of 200 μg/ml. Results presented here extend these observations to 23 different cell types which are unaffected by 50 μg/ml. In another series of recent experiments, Litwin (7) compared the effect of pure reagent grade gentamicin and gentamicin for human use (Garamycin) on the growth of human diploid lung fibroblasts. He found the tissue culture grade (pure) antibiotic to be free from toxicity at 100 μg/ml. However, some additives of Garamycin (see above) proved to be detrimental to cell growth, suggesting that Garamycin should not be used for cell culture purposes.

Gentamicin is stable to extremes in *p*H and temperature, is free from apparent cytotoxicity at bactericidal and antimycoplasmal levels, does not affect stability or replication of viruses, and does not influence the detection or synthesis of interferon. These characteristics indicate that gentamicin may be uniquely suited for tissue culture and virus studies.

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